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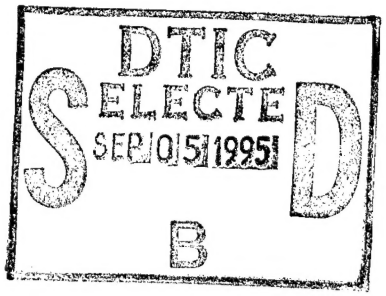

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13. ABSTRACT *(Maximum 200 words)*

The intent of this research is to define the structure and function of a highly conserved protein motif whose ectopic expression causes leukaemia, lymphoma, and mammary carcinoma. This protein motif, called an ankyrin or ANK repeat has been found in a diverse set of proteins in bacteria, plants, fungi, insects, and vertebrates. The ANK repeat is 33 amino acids long and is always tandemly repeated. The analysis that has been carried out indicates that the ANK repeats are required for the activity of the proteins in which they reside, but little progress has been made in defining their function. Deletions within the ANK repeat domains of several proteins suggest that the repeat may be involved in protein:protein interaction.

We are identifying the critical residues and the biological function of the ANK repeats found in two yeast transcription factors. Using yeast allows us to simultaneously exploit genetic screens, gene replacement, biochemistry and immunochemistry in this analysis. A thorough investigation of these repeats in a simple system will provide insight and testable predictions which will augment and expedite their analysis in higher cell ANK proteins. It will also provide valuable reagents for identifying other ANK repeat proteins and defining their functions.

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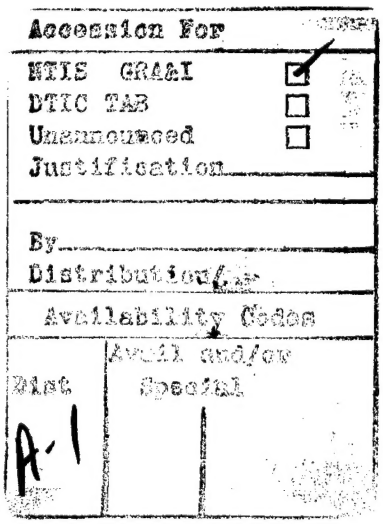

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INTRODUCTION

Ankyrin (or ANK) repeats were first identified in Swi6, a budding yeast protein involved in cell cycle regulated transcription, and in fission yeast homolog of Swi6 (Cdc10) (5). The repeats are called ankyrin repeats because ankyrin, a cytoskeletal protein, contains 22 tandem repeats of this 33 amino acid sequence (18). ANK repeats have also been found in other transcription factors, trans-membrane signalling molecules, toxins, viral host range proteins, and other proteins of unknown function (2). Most recently small ANK repeat proteins have been identified that bind to and inhibit cyclin-dependent kinases (7,11) and the ectopic expression of ANK domains have been shown to leukemia and breast cancer (10,17,20,21).

The number and position of ANK repeats within a protein varies, but they are always found in multiple copies. It is possible that ANK-containing proteins are related to each other, in fact it seems likely that they are all related in some fundamental way, however that is pure speculation at this point. A thorough investigation of the role of the ANK repeats in any one of these proteins will allow us to make educated guesses about the role of the repeats found in other proteins. This would be particularly useful for understanding and overcoming the malignant transformation caused by ANK repeat proteins in murine and human cells.

The consensus sequence of the ANK repeat for the Swi6 family of proteins is shown in Fig. 1, however the consensus within a given repeat, will also vary. Aside from a few relatively invariant residues, there is considerable sequence heterogeneity within the repeats. In addition to the actual sequence conservation, there is some further conservation of hydrophobic and charged residues within the repeats. These could form short amphipathic alpha helices (18), and circular dichroism studies of the ANK domain of ankyrin confirm that it is 30% alpha helical (9).

Despite their persistent occurrence in tandemly repeated arrangements, single mutations in one repeat can result in dramatic loss of activity. The glp-1 protein contains six ANK repeats, however, six different single amino acid substitutions within the ANK repeat domain of glp-1 have been found that cause a temperature sensitive loss of function phenotype (16). A lysine substitution for the most conserved leucine in one ANK repeat of Swi6 also dramatically reduces Swi6 function (1).

There is some evidence for divergence of function in the repeats of ankyrin. Ankyrin is a cytoskeletal protein that binds several proteins, including anion exchange protein and tubulin. The ANK repeat domain is required for binding to both proteins, and there is some evidence that specific repeats within ankyrin are more critical for binding to anion exchange protein than others (9,18). Deletion analysis of several ANK repeat-containing proteins has suggested that the repeats are required for association with other proteins which do not themselves contain repeats (15,25). However, these experiments have all involved large deletions which may cause significant conformational changes outside the repeat domain. Direct contact has not been demonstrated between ankyrin repeats and other proteins, nor have any homologies been observed among the diverse group of proteins to which ANK proteins are known to bind. It is possible that the repeats play an indirect role, by allowing the protein to

adopt a conformation that promotes binding to other proteins, or they may bind through a common intermediate that has not yet been identified.

The experiments that we are carrying out are designed to determine how many functional repeats exist in Swi4 and Swi6, and to identify the critical residues in the repeats of both proteins. We are also defining the biological function of the ANK repeats of both Swi4 and Swi6 through a series of genetic, immunochemical and biochemical assays. Swi4 and Swi6 are members of an evolutionarily conserved family of transcription factors. In budding yeast, they control the expression of the G1 cyclins, which are the rate limiting components of the kinase complex which controls the G1 to S transition. Swi6 complexes with another member of this family of transcription factors, Mbp1, to activate transcription of most of the genes whose products are involved in DNA synthesis. Swi4/6 and Mbp1/Swi6 mediated transcription occurs primarily during the late G1/early S phase of the cell cycle via promoter elements called SCBs and MCBs. The ANK repeats of Swi4 and Swi6 are required for the activities of both proteins, but their actual function is not known.

RESULTS

The purpose of this investigation is to define the function of the ANK repeats in Swi4 and Swi6. We are exhaustively mutagenizing the ANK repeats of Swi4 and Swi6 to identify the critical residues within each repeat. We have planned a series of genetic screens and biochemical assays to screen these mutants and identify the in vivo and in vitro functions that they effect. Partially defective mutants, and mutants that have lost specific functions will be used to identify interacting gene products using suppressor analysis. We are also generating monoclonal antibodies that recognize either Swi4, Swi6 or all ANK repeats. The precise binding specificity of these antibodies will be identified, and their ability to interfere with specific biochemical functions will be tested. Antibodies that recognize general features of the ANK repeats will be used to identify other ANK proteins of yeast and humans.

Our specific Technical Objectives are to:

1. Generate monoclonal and polyclonal antibodies that recognize either Swi4, Swi6 or all ANK repeats and use them to screen for other ANK repeat-containing proteins.

2. Exhaustively mutagenize the ANK repeats of Swi4 and Swi6, and identify the crucial residues for their activity.

3. Develop a battery of genetic screens to identify mutations that cause loss or deregulation of known Swi4 and Swi6 functions.

4. Perform *in vitro* assays to correlate mutant phenotypes with known biochemical functions.

5. Identify second site suppressors, either within the Swi protein, or within associated proteins.

We have made significant progress on objectives one, two and five. Our results are sumarized below.

Task 1. Generating monoclonal antibodies that recognize Swi6 and the ANK repeat region of Swi6.

We have generated monclonal antibodies to full length Swi6 and the the ANK domain of Swi6, as described in my research proposal. Table 1 summarizes the monoclonal cell lines that have been generated and the extent to which they've been characterized.

Table 1. Monoclonal antibodies generated in this study.

		Antibodies positive for use in:		
Fusion Number	Immunogen	ELISA	Western Blot	Immunoprecipitation
1	Swi6 repeat domain	24	11	1
2	Swi6 repeat domain	57	14	0
3	Swi6	33	0	0
4	Swi6	35	4	2

Monoclonal production using RBF/DnJ mice was carried out as described (24). ELISA, Western Blot and immunoprecipitation techniques were carried out using standard protocols (14).

We are in the early stages of characterizing these antibodies, and using them for analyzing Swi6 and ANK repeat function. The results of these studies will be described in later progress reports. So far none of the antibodies that we have generated react with other *S. cerevisiae* proteins besides Swi6. Our efforts to screen these antibodies for cross reactivity with the Cdc10 protein of *S. pombe* have been inconclusive.

Task 2. Defining critical residues within the ANK repeat domain of Swi6.

The first step in the analysis of the ANK repeat domain of Swi4 and Swi6 was an alignment and comparison of the ANK repeat domains of the eight known members of this family of transcription factors. Fig. 1 shows this alignment which I have submitted for publication (3)in a review on this subject.

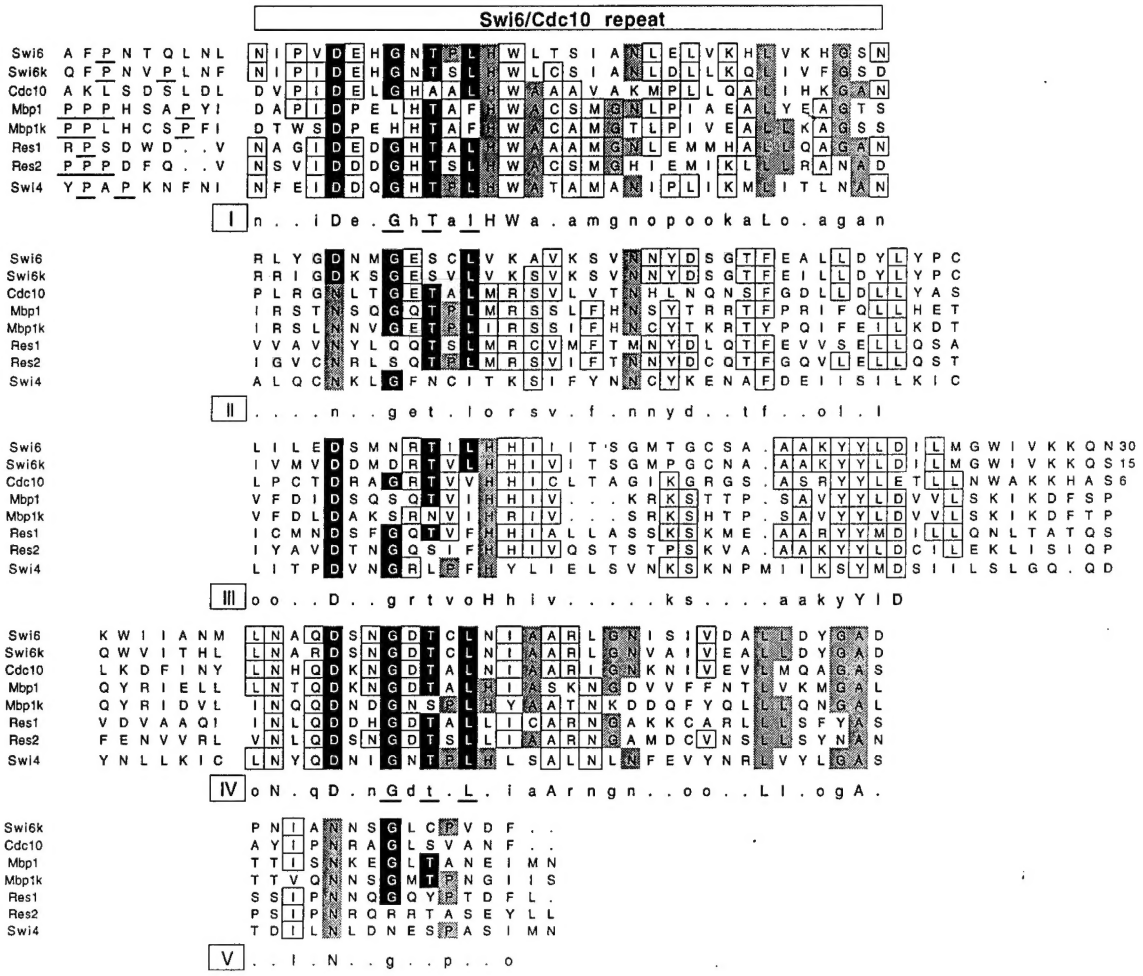

Figure 1. Aligment of the Swi6/Cdc10 repeat domain shows the residues common to all five repeats in black and the residues conserved in a subset of the repeats in grey. In addition, boxed residues indicate amino acids that are conserved in at least four family members but only within a single repeat. The consensus sequence for individual repeats is shown below the alignment. Capital letters denote identity in all eight family members, lower case residues are conserved in at least half of the sequences, and a lower case o denotes positions that are always hydrophobic. The high propensity for proline residues preceedindg the Swi6/Cdc10 repeat domain is emphasized by underlines. Also underlined in the consensus sequence of repeats I and IV, are the positions of the six alanine substitutions in Swi6 that inactivate Swi6 and disrupt DNA binding. This alignment represents the contiguous sequences of all eight family members except for small nonhomologous inserts that exist in three of the proteins after repeat III. The length of the omitted sequence is noted.

It is apparent from this alignment (Fig. 1) that there is considerable homology throughout the ANK region (black and grey shaded residues). This conservation suggests the existence of 4.5 repeats. There is further homology (boxed residues) within a given repeat, which makes the consensus sequence for each repeat slightly different. The homologies evident from this alignment make it possible for us to interpret the mutations that we have generated in Swi6.

Mutagenic PCR (6) was used to produce three different library's of ANK domain plasmid-borne mutants of Swi6. These were made with different PCR conditions to produce different frequencies of mutations. We have screened for all types of defects in Swi6, but have focussed most of our attention on temperature sensitive mutants that are very defective in *HO:lacZ* transcription at 37° and active at 25°. These plasmid-borne mutants were tested to make sure the ts mutation was carried by the plasmid and that the Swi6 protein was still accumulating at the high temperature. The latter test insured that the mutations are affecting function rather than just the stability of the protein at high temperature, then these mutants were sequenced. A summary of this work is provided as Table 2.

Table 2. Mutagenesis of the ANK domain of Swi6.

Library	Bd1681	BD1824	BD1825
$MnCl_2$	.5 mM	.25 mM	.1 mM
Complexity	300	1,000	1,000
Total Mutants Isolated	85	7	18
Temperature Sensitive	18 (8)	5 (5)	7 (7)
Null	52	2	7
Partial Activity	15	0	4

Each library was fully screened (>4 x complexity). The numbers in parentheses represent the number of these mutants that have been sequenced.

Most of the mutants we have characterized so far contain more than one mutation and many of these reside in residues whose importance to function was unknown. Thus, from this analysis we have more evidence of the importance of unique residues in individual repeats than we have of the importance of consensus residues.

To test directly whether the consensus residues in each of the four complete ANK repeats are critical for function of Swi6, we made four site-directed mutants in which the conserved core of each repeat: G-T-L, was changed to A-A-A. We then measured *HO:lacZ* expression by filter (4) and liquid (19) assays and the liquid assay results are shown in Table 2. The consensus residues in the fourth repeat are clearly the most critical

for function, but mutations in each of the four conserved cores cause a loss of function when mutated to alanines. The fact that the conserved residues that were changed are important for Swi6 activity suggests that this region contains four functional repeats. The importance of the fifth half repeat has not yet been investigated.

Table 3. All four ANK repeats of Swi6 contribute to *HO:lacZ* activation.

PLASMID	*SWI6* ALLELE	*HO:lacZ* activity
BD1378	Swi6 WT	12.64
BD1819	Swi6 3A-1	1.99
BD1820	Swi6 3A-2	0.57
BD1821	Swi6 3A-3	0.38
BD1822	Swi6 3A-4	0.05
None	--	0.00

Wild type and Swi6 mutants bearing three alanine substitutions in ANK repeat 1 (3A-1) through 4 (3A-4) were expressed on plasmids in a *Δswi6 HO:lacZ* strain (BY600). These strains were assayed for *HO:lacZ* expression as described (4,19).

These triple mutants were screened only for loss of function using the *HO:lacZ* reporter, so other Swi6-dependent activities will have to be assayed. These include activation of MCB regulated promoters, alfa factor recovery, and a new phenotype, which is hypersensitivity to mutagens. Once the spectrum of Swi6-dependent activities are tested, we will know if mutations in each of the repeats has an equivalent impact on all Swi6 functions, or if they differ. If each repeat has a different primary function this should be evident from this analysis. If individual repeats show a differential importance in any of these assays, this information will be useful for screening for other mutations in the critical residues in each repeat.

Task 5. Suppressors of temperature sensitive ANK mutants.

Suppression of leaky or temperature sensitive mutations has been widely used to identify other proteins that interact with the mutant protein. The rationale is that slight defects or instabilities within an interaction domain can be suppressed either by compensating mutations, or by overproduction of the binding partner. Both G1 and G2 cyclins have been identified in this way (13,23), as well as many other interacting yeast proteins (8,12).

We have generated three libraries of Swi6 ankyrin repeat mutants, with complexities of about 2000 mutants each, using mutagenic PCR (see Task 2). We have screened these libraries and selected 17 mutants of Swi6 that are ts for *HO::LacZ* transcription and that continue to accumulate Swi6 protein at the high temperature. This was a necessary second criteria because if the protein was degraded, overproduction of its binding partner could not suppress its defect. We've integrated eight of these mutants into the yeast genome for further study and activity measurements (22). We have also screened for high copy suppressors of the ts phenotype for two of them: swi6-B38 and

swi6-B19. Two more, swi6-wh6 (a double) and swi6-4-10 (a single) show high degree of reversion and are unsuitable for suppressor screens.

We have isolated two high copy suppressors in this analysis, one of which bypasses a *swi6* null mutant and therefore must be activating Swi6 target promoters in a Swi6-independent manner. This suppresor is not relavent to the analysis of Swi6 function, but may be useful in other studies. The second high copy suppressor is allele specific. It suppresses ANK mutant B19, but it does not suppress *swi6* deletions or other mutations elsewhere in the protein. This overexpressed gene is a good candidate for encoding a protein that interacts with the ANK domain of Swi6. We are currently sequencing this gene, and fully characterizing its allele specific suppression.

CONCLUSIONS

Some portion of the ANK domain of Swi6 is accessible to antibodies in the native state. Swi6 is an antigenic protein, as are the ANK repeats in isolation. The fact that at least one monclonal antibody generated against the ANK domain can be used to immuneprecipitate Swi6 from yeast extracts suggests that some portion of the ANK domain is accessible in the native state. Not all of the antibodies have been assayed for immuneprecipitation as yet. This work is underway. We had hoped that the ANK repeat fragment of Swi6 would be soluable and properly folded as produced in E. coli, but this is not the case. This means that this protein preparation is not ideal for generating antibodies to native structure. Thus we have shifted to using full length Swi6, which we know is functional as the immunogen.

There are at least four functional ANK repeats in Swi6. The fourth repeat of Swi6 is the most critical for Swi6 function, but the conserved core of each repeat is important for Swi6-mediated activation of HO:lacZ transcription. It will be important to determine at what level the defect manifests itself. It will also be interesting to see if individual repeats have different impacts on other Swi6-dependent processes (MCB activation, α factor recovery, etc.).

Many residues within the ANK domain are critical for function. ANK domain mutations have been identified that cause Swi6 to be temperature sensitive for function. Mutants across the spectrum of severity have been isolated from this screen. We have focussed upon temperature sensitive ones, but many non-conditional null mutants have also been isolated. These will be characterized in the future. Judging from the analysis so far it is clear that the ANK domain of Swi6 contains a large number of residues that are critical for its function.

A candidate ANK domain interacting protein has been identified by high copy suppression. Allele-specific suppression has been used to identify many interacting proteins. The suppressor we have identified selectively suppresses mutations within the ANK domain of Swi6. Our intent is to identify this gene and test the prediction that it binds to the ANK domain of Swi6 using immunoprecipitation. Our monclonal antibodies and the starting mutants will be valuable tools for this analysis.

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